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(57) Abstract

A physiologically-acceptable formulation comprising two or more of: (a) an obligate anaerobe capable of converting lactic acid; (b) a facultative anaerobe; and (c) one or more enzymes capable of degrading starch or fibre to assimilable material. These components are useful to prevent or inhibit acidosis, especially in ruminants, and can thus promote weight gain or milk production.

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Micro-organisms, Enzymes, and Their Use

Field of the invention

This invention relates to micro-organisms, enzymes and their use. In particular it relates to the use of micro-organisms and/or enzymes to improve or enhance the performance, e.g. growth or weight gain, of farm animals and the value of the feed they receive.

Background of the invention

WO-A-9210945 (and the corresponding US Patent No. 5432074, the content of which is incorporated by reference) describe the utility of micro-organisms and enzymes in enhancing the value of prepared silage. Micro-organisms can also be used to enhance animal performance, as described in WO-A-9313786 (and the corresponding US Patent Application Serial No. 08/255,657, the content of which is incorporated by reference).

In the ruminant, the use of enzymes has largely been restricted to the pre-treatment of forages to improve the preservation of the forage, to reduce nutritive loss through effluent production, and to enhance intake and nutritive value. Similarly the use of micro-organisms has been restricted to those that have their ecological niche in the lower intestine rather than in the rumen.

Up to now it has always been considered that the fermentation in the rumen functions close to maximal efficiency on the assumption that "it is unlikely that man can improve on what has been the product of many thousands of years of the evolutionary process". However, man's ability to change the animal's environment and nutrition have taken us a long way from the normal condition of the grazing animal. Other animals have fermentions in their gastro-intestinal system and, although they do not have the complex system of the stomach found in the ruminant, and

such fermentations are less efficient, they do occur in parts of the gastro-intestinal tract of other animals, notably, the hind gut of pigs, the caeca of rabbits, equidae, and birds.

In ruminant animals, under normal grazing conditions, the feed is predominantly vegetable fibre. Other feeds are however commonplace, particularly where husbandry practice becomes intensive and concentrated rations are required to be delivered to penned or stalled animals. Such feeds include brans from maize, rice and wheat, whole crop maize, sorghum, wheat, oats and barley. By-products include palm kernel, and citrus pulp and even paper. At the other extreme, high starch diets include whole and rolled grains of wheat, maize barley and sorghum. Each presents its own particular problems to the organisms present in the rumen which, under normal grazing regimes, are and have evolved to be admirably adapted to the extraction of energy from grass. However, the modern practice of feeding by-products, higher energy forages (e.g. whole crop) and high energy grains has lead to conditions where the rumen microbial system can become distorted and inefficient. A prime example of this is the situation where a rapid change to high energy high starch rations results in the proliferation of the organism *Streptococcus bovis*. This organism, one of the few lactic acid-producers that can ferment starch, will produce so much lactic acid that the pH will drop considerably in the rumen. It acute cases this leads to a drop in blood pH and death. In sub-clinical forms, the organisms of the rumen, especially those that are involved in fibre degradation, are reduced to insignificant numbers. This is particularly true of the rumen fungi. Fibre degradation, rumen fermentation and the general health of the animal are impaired.

These problems in the raising of livestock have long been known to the farmer who has sought to contain them by

practice and good husbandry, together with the use of antibiotics such as Monensin and Tylosin fed, usually at sub-clinical doses, prophylactically. However, the problems associated with abnormal feeding regimes are often exacerbated by other problems related to feeding intakes. This may often be found and referred to as "stress". This is particularly exemplified in the ruminant during times of fasting and subsequent engorgement. This is prevalent at the onset of spring or the onset of extremely hot weather which depresses intake with a compensatory elevation of intake on the onset of more temperate conditions. On all these occasions increased nutrients, particularly starch, may tip a condition of sub-clinical acidosis into an acute phase followed by death.

US-A-4138498 and WO-A-9113146 disclose strains of the obligate anaerobe *Megasphaera*, to prevent or minimise acidosis in ruminants. For example, *M. elsdenii* may ferment lactic acid in preference to simple sugars. It is proposed to use such an organism in combination with other organisms, to produce propionic acid in the rumen.

A further problem is associated with nutrition in the practice of ruminant husbandry. In the feed of high starch-containing feeds, e.g. cereals such as wheat, barley, maize or sorghum, much of the starch can by-pass the digestive system of the ruminant altogether. One solution has been to roll, treat with chemicals, or to steam-flake the grain. This only serves to exacerbate the problems associated with acidosis. Similarly, enzyme treatments, whether with fibre, starch, or protein-degrading enzymes, can push the ruminant into an acidotic condition when used as a treatment with steam-flaking or as a stand-alone treatment.

The task for the modern nutritionist is therefore to maintain the natural rumen fermentation process, increase

the nutritive value of the feed, and increase starch utilisation, while preserving the natural balance of the organisms of the ruminal fermentation. The removal of prophylactic antibiotics is also thought to be desirable, in the interests of environmental ecology.

Summary of the invention

The present invention is based at least in part on the discovery that the presence of small amounts of oxygen in the rumen may also cause a disruption of rumen condition and the loss of rumen bacterial. Another aspect of this invention lies in the realisation that enzymes can be used to realize the nutritive value of certain feeds, especially when microorganisms are used to prevent acidosis.

One aspect of this invention lies in novel enzyme formulations. Another aspect provides a combination of micro-organisms and optionally also enzymes to maintain a natural fermentation, and to allow the better use of all feeding stuffs, especially starch, without causing damage to the fermentation or to the health of the animal. The invention proposes that the use of one or more organisms on their own or in conjunction with selected enzymes may, when added to the feed, or administered separately, promote the well-being of the animal, preserve the natural fermentation, and promote growth, feed conversion and performance, while permitting the removal of certain antibiotics from the feed.

Description of the invention

For the purpose of the present invention, the biological ingredients and additives that will effect the desired improvement in the husbandry of the animal and the utilisation of the feed can be divided into two groups, i.e. micro-organisms and enzymes. By way of example, when using microorganisms, treatment of acute or sub-acute acidosis usually requires that the treatment is applied

prophylactically on a daily basis. Single treatments will result in only a partial solution of the problem.

Enzymes and micro-organisms may be administered in any manner that maintains the biological activity of the active ingredients so that they reach the rumen in an active state. They may be included in complete rations or in water provided for drinking. This achieves the desired result of dosing the rumen in a consistent manner over a prolonged period, rather than on a batch application method. However, a manual or similar dosing method does have applications, for example for hand-reared animals, and for animals under veterinary supervision.

The maintenance of the desired condition of the rumen may be achieved by the addition of one or more organisms that are normally found in the rumen. For example the re-establishment of fibre digestion can be achieved by the addition of ruminal fibre-degrading bacteria and fungi. However the ruminal pH must first be raised to a pH close to 6.5 and be maintained there before this can be done. This can be achieved by adding one or more organisms capable of metabolising lactic and other acids. These are exemplified by the genera *Propionibacter*, *Selenomonas*, *Velionella*, and *Megasphaera*. Certain *Bacilli* may also utilise lactic acid without VFA production. Of these organisms, the *Bacilli* and *Megasphaera* may prove the organisms of choice since their ability to metabolise lactic acid may be independent of fermentable carbohydrate in the rumen, and other organisms may use fermentable carbohydrates in preference to lactic acid. Thus they may be less suited to high acid high carbohydrate found in acidosis as it approaches the clinical stage. Many of these organisms are obligate anaerobes and may be less effective where the rumen is insufficiently anaerobic.

The use of such organisms to maintain rumen condition and pH can themselves be enhanced by the use of facultatively anaerobic organism of non-ruminal origin as well as those from the rumen. In such cases, organisms can act by ensuring the anaerobicity of the rumen, promoting the natural flora and enhancing the effects of the oxygen-intolerant lactic acid utilisers. Such facultative organisms include lactic acid bacteria such as *L. acidophilus* and yeasts.

The organisms can be added as an oral drench and such application will last for a number of days. However, for complete treatment, the application of the organism is advantageously applied on a daily basis at doses of between 1×10^4 and 1×10^{10}. There may be a need to resort to the higher doses where the product is being introduced to the animal for the first time or where the levels of stress are particularly obvious. The most satisfactory delivery method is as a liquid dispensed on to the feed immediately before consumption. Other methods such as powder application are also suitable, providing the essential prerequisite of inoculating the rumen is achieved.

Such applications will maintain the condition of the rumen and ensure good conversion of the feed into microbial and ultimately into animal protein.

Notwithstanding the potential effects of adding fibre-degrading organisms to the rumen, enzymes may be preferred for a more rapid response. Fibre utilisation may be improved by the use of cellulases, hemicellulases, and enzymes degrading oligosaccharides as low as disaccharides. Starch utilisation can be enhanced by the use of amylases and/or amylopectinases. A significant improvement can be found where proteinases are used to degrade that protein encapsulating the starch granule, thus permitting greater access of the enzymes to the starch. In all these cases,

the condition of the rumen needs to be maintained and the use of the enzymes with one or more of the organisms is preferred.

By way of example, enzyme treatment is particularly suitable for whole crop forage which includes a high starch content, for the maintenance of rumen condition. In the UK at least, the growing of wheat oats and barley as a feed for ruminants is increasing where maize cannot be grown. These cereals are normally harvested with a high dry matter and treated with urea. An alternative treatment is described in WO-A-9210945. In either case, a formulation of the invention is then administered with the treated whole crop silage.

Whole wheat, other cereal or grass is first ensiled with products that will preserve the material, enhance its nutritive value, and prevent subsequent aerobic spoilage. Such products generally will contain one or more lactic acid-producing organisms also capable of producing acetic acid and or propionic acid. Enzymes will be typically of the xylanase (pentosinase) type capable of carrying out the initial stage of fibre degradation and the separation of the polysaccharides from the lignin matrix. Such products are exemplified in WO-A-9210945 but here are used on whole crop materials that are typically higher than 35% dry matter, of which 15-40% may be starch.

The enzyme and micro-organisms will have completed much of their effects well before the fodder is ingested.

The whole crop may now be used as a carrier for the enzymes that are required for a further enhanced degradation in the rumen. The enzymes that cannot be used in the preparation of the silage, because of the enhanced risk of effluent production with a subsequent loss of nutrients, may now be added to the fodder immediately prior to ingestion. On

being carried into the rumen, they will function under the controlled conditions that are present there.

Although the formulations may be tailored to each specific type of forage, the enzymes for whole crop wheat will be centred on the two main cellulolytic enzymes in combination with xylanases, arabinosidases, glucosidases, xylosidases and similar enzymes. Specific suitable enzymes are given in Example 2.

Especially in the treatment of whole grain cereals such as wheat, enzyme formulations are based on the need to remove fibrous husks, loosen the starch grains, remove the starch grains' protein coat and, especially, assist in at least partial starch digestion. Where starch is fed in high quantities, it is often the case that only organisms such as *Strep. bovis* can utilise the starch. Many other rumen VFA-producing organisms will not do so; this gives rise to a proliferation of lactic acid producers at the expense of other organisms. The use of amylases, releasing glucose, and glucose polymers will permit the non-amylase producers to compete more effectively thus reducing somewhat the slide into a predominantly lactic fermentation. Suitable enzymes are given in Example 3.

As with wheat, barley and oats, the cereals maize and sorghum need to be treated either by rolling or flaking to enhance performance. In addition, with these grains, maize in particular, the individual starch granules are held in a protein matrix. This protein is precipitated and partially denatured when the grain becomes dry, typically at moisture contents lower than 28% moisture. This denatured and relatively indestructible protein coat prevents the full utilisation of the starch in the rumen. Thus even cold-rolled grains can be enhanced by enzyme treatment of the proteins in the grain by the use of additional enzymes in the rumen.

Further improvements will be found in the treatment of whole grain where it is treated first as indicated for whole crop and subsequently used as a carrier for additional enzymes into the rumen. Here, as with wheat, the inclusion of amylase with the protease will give rise to sugars for fermentation and again help to counter any drift to a lactic fermentation. Suitable enzymes are given in Example 4.

Where the ration is consistently high in fibre, the opportunities for manipulation in the rumen are different. Here the problems relate to the rate at which food is taken in and passed through the rumen and out into the lower parts of the digestive tract. The task is to optimise the fermentation in the shortest dwell time in the rumen. Fibre prepared using the type of additive described above for the preparation of whole crop forage will start with an added advantage; nevertheless, with silages of between 15 &35% (c.f. 45% for whole crop), considerable improvements may be obtained.

Enzyme preparations may be administered direct to the animal or more conveniently to the fodder at the time of ingestion. The enzyme activities will in this case be guided to that group of enzymes capable of fibre degradation but will include the group of enzymes more normally associated with pectin degradation. Suitable enzymes are given in Example 5.

The following Examples illustrate the invention.

Example 1

The treatment of sublicinical acidosis can be achieved by the addition of a viable lactic acid organism, together with another organism capable of reducing the oxygen tension in the rumen. Viable cells of a lactate user e.g.

Megasphaera elsendenii and an oxygen user e.g. *Lactobacillus acidophilus* at daily dose rates of between 1 x 10^3 and 1 x 10^{11} and between 1 x 10^4 and 1 x 10^{10} achieve satisfactory control of the acid production in the rumen whether produced from starch or other fermentable materials. Dose rates of 5 x 10^8 and 1 x 10^9 for *Megasphaera* and *Lactobacillus* are currently being used in an American feed yard with animals on high starch rations under intensive conditions.

Example 2

A formulation contains the following enzyme activities:

1,3-(1,3;1,4)-ß-D-glucan-3(4)-glucanohydrolase	EC 3.2.1.6
1,3-(1,3;1,4)-ß-D-glucan-4-glucanohydrolase	EC 3.2.1.4
ß-D-Glucoside hydrolase	EC 3.2.1.21
1,4-α-D-Glucan glucohydrolase	EC 3.2.1.1
1,4-ß-D-Xylan xylanohydrolase	EC 3.2.1.8
Xylan 1,4-ß-xylosidase	EC 3.2.1.37
α-L-Arabinofuranoside arabinofuranohydrolase	EC 3.2.1.55

The addition of this formulation of enzymes at activities of between 60 and 600 units per animal per day gives increases of milk yield of 1-5 litres per day.

These enzymes may be added on their own where starch activities are low. Good results are obtained. When the starch levels are high, the enzyme formulation is used in conjunction with one or both of the organisms in Example 1.

Example 3

A formulation contains four of the activities tabulated in Example 2, i.e. EC 3.2.1.1, EC 3.2.1.8, EC 3.2.1.37 and EC 3.2.1.55, plus bacillolysin EC 3.4.24.28.

Whole grain wheat is treated with this formulation plus one or both of the micro-organisms given in Example 1 and fed within a short period of time, to allow the enzymes to continue to function in the rumen. This procedure gives feeding values close to those of rolled or crushed grain.

Example 4

A formulation contains the activities listed in Example 2, plus bacillolysin EC 3.4.24.28.

Whole grain maize and sorghum are treated with this formulation, and one or both microorganisms, in the manner described in Example 3.

Example 5

A formulation contains the activities tabulated in Example 2, plus poly(1,4-D-galacturonide) glucanohydrolase EC 3.2.1.15 and poly-(methoxy-L-galacturonide) lyase EC 4.2.2.10.

This formulation is dispersed on forage and administered to cows, optionally with one or both of the organisms of Example 1, to increase milk yield.

The application of enzymes directly and equally dispersed over the forage is an important aspect of the application process. In the rumen, proteins are rapidly degraded in a hostile environment and the even distribution of the enzyme on the fibre followed by a sufficient time (about 30 min) for the enzyme to bind to the fibre ensures both survival and continued activity in the rumen after ingestion.

Preparations and their activities will vary according to the fodder used and its proportion in the diet but typically will give rise to an increase in growth rate over

controls or similarly an increase in 1 to 2 litres of milk in a dairy cow.

Amylase in the case of fibre digestion is likely to of greatest value in leguminous silages. In all cases the rate of fibre digestion is best achieved by the maintenance of a good fibre digestion ability in the rumen. This is achieved in conjunction with one or more of the organisms indicated in earlier Examples.

CLAIMS

1. A physiologically-acceptable formulation comprising two or more of:

(a) an obligate anaerobe capable of converting lactic acid;

(b) a facultative anaerobe; and

(c) one or more enzymes capable of degrading starch or fibre to assimilable material.

2. A formulation according to claim 1, comprising at least a combination of the obligate and facultative anaerobes.

3. A formulation according to claim 1 or claim 2, wherein the facultative anaerobe is selected from yeast, and lactic acid bacteria.

4. A formulation according to any preceding claim, wherein the or each organism is capable of metabolising acids and thus maintaining the rumen pH at or about 6.5.

5. A formulation according to any preceding claim, wherein the obligate anaerobe is selected from *Propionibacteria, Velionella, Selenomonas, Megasphaera* and *Bacillus licheniformis*.

6. A formulation according to any preceding claim, which comprises at least the one or more enzymes.

7. A formulation comprising the following enzyme activities:

1,3-(1,3:1,4)-β-D-glucan-4-glucanohydrolase

1,4-β-D-xylan xylanohydrolase and

α-L-arabinofuranoside arabinofuranohydrolase

having the respective Enzyme Commission (EC) numbers 3.2.1.4, 3.2.1.37 and 3.2.1.55.

8. A formulation according to claim 1, which additionally comprises the activity of EC 3.2.1.6.

9. A formulation according to claim 7 or claim 8, which additionally comprises the activity of EC 3.2.1.21.

10. A formulation according to any of claims 7 to 9, which additionally comprises the activity of EC 3.2.1.1.

11. A formulation according to any of claims 7 to 10, which additionally comprises the activity of 1,4-β-D-xylan xylanohydrolase EC 3.2.1.8.

12. A formulation according to claim 6, wherein the one or more enzymes are selected from those defined in any of claims 7 to 11.

13. A formulation according to any of claims 6 to 12, which includes a proteolytic enzyme for example Bacillolysin EC 3.4.24.28.

14. A formulation according to any of claims 6 to 13, which includes a amylolytic enzyme such as EC 3.2.11.

15. A formulation according to any of claims 6 to 14, which comprises cellulose 1,4-β-cellobiosidase EC 3.2.1.91.

16. A formulation according to any of claims 6 to 15, which additionally comprises the activity of EC 3.2.1.28.

17. A formulation according to any of claims 6 to 16, which additionally comprises the activity of EC 3.2.1.15.

18. A formulation according to any of claims 6 to 17, which additionally comprises the activity of EC 3.2.1.10.

19. A formulation according to any of claims 6 to 18, wherein the enzymes are bound to a substrate or a carrier such as starch such that proteolysis of the enzymes is inhibited.

20. An animal feed which comprises a formulation according to any preceding claim.

21. An animal feed according to claim 20, which comprises starch and a formulation as defined in claim 14.

22. An animal feed according to claim 20, which comprises whole grain cereal.

23. An animal feed according to claim 20, which comprises whole crop silage.

24. An animal feed according to claim 20, which comprises maize silage.

25. An animal feed according to claim 20, which comprises high fibre forage, e.g. grass silage.

26. An animal feed according to any of claims 20 to 25, which comprises concentrated and pelleted feed.

27. A method for preparing an animal feed according to any of claims 20 to 26, which comprises treating the feed or silage with the components of a formulation as defined in WO-A-9210945, and adding the components of a formulation according to any of claims 1 to 19.

28. A method of treating livestock to maintain or increase weight or production, which comprises administering to the livestock components (a) and (b), (a) and (c), or (a), (b) and (c) as defined in any of claims 1 to 19.

29. A method according to claim 20, which comprises administering the organisms and enzymes separately.

30. A method according to claim 20, which comprises administering the active ingredients as a single dose.

31. A method according to any of claims 28 to 30, wherein the organisms are administered continuously (daily) and at increased doses up to 1×10^{11} under adverse conditions.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A23K1/00 C12N1/20 C12N9/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A23K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO,A,93 20714 (SSV-DEVELOPMENT OY) 28 October 1993 see page 1, line 5 - page 10, line 21 see examples 1-7 see claims 1,3,28,29,37-39 ---	1-11,15, 19-27
X	DATABASE WPI Week 9038 Derwent Publications Ltd., London, GB; AN 90-285864 & JP,A,02 200 195 (NIPPON SHOKUHIN KAK KK) , 8 August 1990 see abstract --- -/--	7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
15 March 1996	22. 03. 96

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO,A,92 10945 (ENZYMATIX LTD.) 9 July 1992 cited in the application see page 4, line 2 - line 35 see claims 1-10	7,20-26
A	see page 5, line 6 - line 36	1-6,27, 28

Y	WO,A,91 15966 (SSV-DEVELOPMENT OY) 31 October 1991 see page 23, line 21 - page 24, line 2 see page 24, line 21 - line 26 see page 30, line 6 - line 17 see claims 1-4,17,18,26,54-67	7,20-26

A	EP,A,0 071 858 (MILES LABORATORIES INC.) 16 February 1983 see page 4, line 24 - page 5, line 4 see claims 1-9	1-5,20

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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